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Atty. Docket No. CRP-008DV

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S): Huston, James S. et al. EXAMINER: J. Ulm

SERIAL NO.: 07/661,070 GROUP NO.: 185

FILED: February 26, 1991

TITLE: PRODUCT AND PROCESS FOR THE PRODUCTION, ISOLATION AND PURIFICATION OF RECOMBINANT POLYPEPTIDES

Honorable Commissioner of Patents
& Trademarks
Washington, D.C. 20231

DECLARATION OF DAVID C. RUEGER

UNDER 35 U.S.C. RULE 1.131

I declare:

1. I, David C. Rueger, hold a Ph.D. in Biochemistry from Duke University. I am a named inventor of the above-captioned patent application and hold the position of Senior Research Director at Creative Biomolecules, Inc., the assignee of the above-captioned patent application.

2. Prior to March 28, 1986, I and my coinventors reduced to practice in the United States the construction and expression of recombinant DNA encoding a fusion polypeptide containing a leader/hinge region, a cleavage site, and a target polypeptide, and the subsequent cleavage of that polypeptide at the cleavage site by a cleavage site-specific protease. The following experiments, performed by Zita Babickas under my direction, demonstrate that a fusion polypeptide having the structure described in the above-captioned patent application is cleaved at the cleavage site nearest the hinge region. These experiments are described in the above-captioned patent application (see Example III, in particular) and in the accompanying Exhibits A and B. The results are shown in Exhibits C-E. These experiments involve fusion polypeptides in which the target protein is calcitonin.

3. Polypeptide 1, described on pages 39-41 of the above-captioned patent application, includes a TRP-LE leader sequence connected at its carboxyl end to a Phe-Pro-Gly hinge, followed by a glutamic acid residue and human calcitonin. As stated in the specification on page 40, lines 1-7, it was desired that the tripeptide hinge would promote cleavage at the Glu residue by providing a flexible residue (proline) and an uncharged "spacer" residue next to the Glu residue. The large leader was used to promote high expression levels. The sequence of polypeptide 1 is provided in the specification on pages 40-41.

4. Polypeptide 2, described on pages 40-41 of the above-captioned patent application, is a smaller polypeptide in which the TRP-LE leader is truncated, a dipeptide Asp-Leu hinge, a Glu cleavage site, and salmon calcitonin analog as a target polypeptide. The salmon calcitonin analog was used because it contained an Asp at position 15 instead of the cleavable native Glu. The sequence of polypeptide 2 is set forth on page 41 of the specification. As stated in the specification on page 41, polypeptide 2 was designed with the goal of obtaining a fusion polypeptide which was cleavable into only 2 fragments, the hook/hinge fragment and calcitonin. Also, the hinge of polypeptide 2 was designed to facilitate cleavage with V-8 protease such that cleavage approached 100% completion.

5. Exhibits A and B, attached hereto, demonstrate the experiments in which polypeptides 1 and 2, respectively, were cleaved with V-8 protease. These experiments were performed and the pages submitted as Exhibits A-E are dated prior to March 28, 1986. Exhibits C, D, and E show the results of these cleavage experiments. Exhibit C shows cleavage of polypeptide 1, in which V-8 protease cleaved the fusion protein into multiple fragments. Exhibit D shows cleavage of polypeptide 2, in which V-8 protease cleaved the fusion protein into the two expected fragments, identified as "hook" (containing the leader and hinge regions) and "SC" (containing the salmon calcitonin analog). Exhibit E

shows the purified salmon calcitonin analog collected from the "SC" peak shown in Exhibit D.

6. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1011 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

David C. Rueger

David C. Rueger

3-18-92

Date

189kam2054/6.AH6

PROJECT Calcitonin 13[redacted] [redacted] polypeptide 1

Continued From Page

Staph cleavages (bulk) of LE-Glu-HC in 4M+6M urea w+w/o DTT

2 ml of C130-pool were dialized overnight vs 6M urea H_2O E, pH:
Concentration assumed ~.7mg/ml as before

code	µl prot	µl buff	mg prot	ratio	µl enzyme
133-A	500	-	.355	1:50	6.7
-B*	330	170	.234	1:50	4.7

another shot of 1:50 enzyme added after 1hr. (4:30 pm) (C133-A,B-2hr+n

Gel samples taken @ 2hr, 2hr+5hr, 2hr+18hr.

12/84 another shot of 1:50 enzyme added after 21 hrs (1:30 pm 9/12) (C133-A,B-2hr+17h
4µl of staph added to A @ 5:30 pm (C133-A-2hr+24[redacted]
+6+[redacted]hr.

code	µl prot	µl buff	µl .6M DTT	mg prot	ratio	µl enzyme
33-C*	200	100	-	.142	1:50	2.84
-D*	200	100	10	.142	1:50	2.84

protein incubated w/ DTT for 2 hrs before enzyme addition - 2mM DTT final conc
extra shot of 1:50 added after 3hrs.
C received 2nd extra shot after 3 hrs. (C133-C-2hr+3hr+n) ≠
D " " " " 18 hrs (C133-D-2hr+18hr+n)

13/84

code	µl prot	µl buff	mg prot	ratio	µl enzyme
33-F*	1.0	.5	.71	1:50	13.4

extra shots after 2hr + 18 hrs (C133-F-2hr+18hr+n)
pH was ~7.1 after 18hr - brought up to pH 7.9 before addition of enzyme

All 4M urea samples were turbid after ~1hr

Yield: 200µl collection (94.6µg injected x .14 (14%) = 13.2µg calc corrected)
peak weight = 7.5% of total = 7.1µg = 53.8%
" OD = .37 ÷ .035/µg = 10.6µg = 80.3%

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Read and Understood By

Rita A. Babickas C.H.

PROJECT Calcitonin 180 Continued From Page

Bulk Staph cleavage of reduced Synthetic SC-asp

2 ml of conc C178-pool made 20mM STT + Urea @ RT for 2 hrs
Dialyzed overnight vs 1L 6M Urea 20mM NH4 Acet E, pH 7.8 (1.3 mg/ml)

4/7/85

(A) 100 µl of protein (assume mg/ml) + 50 µl 20mM NH4 Acet E, pH 7.8
+ 3 µl staph (Pierce .64 mg/ml) 1:50 excess @ 9:50 (1:70 real)
collected @ 45' + 1¼ hrs

(B) 200 µl of protein (.26 mg) + 100 µl 20mM NH4 Acet E, pH 7.8
+ 2.7 µl staph (Pierce .64 mg/ml) 1:150 excess @ 12:10
collected @ 30', 1 hr + 1½ hrs

(C) 200 µl of protein (.26 mg) + 100 µl 20mM NH4 Acet E, pH 7.8
+ 2.7 µl staph (Pierce .64 mg/ml) : 1:150 excess @ 2:00
collected @ 30', 1 hr + 1½ hrs.

(D) 200 µl of protein + 100 µl 20mM NH4 Acet E, pH 7.8
+ 2 µl staph (Pierce .64 mg/ml) 1:200 excess @ 4:35
left 4°C after ~2 hrs

Yield: ~90 µg of cSC / collection on LC (100 µl of each digest)
∴ 90 µg / 10 µl conc C178-pool
∴ 9.5 mg cSC / 10 gr cells.
~ 1 mg cSC / g. cell

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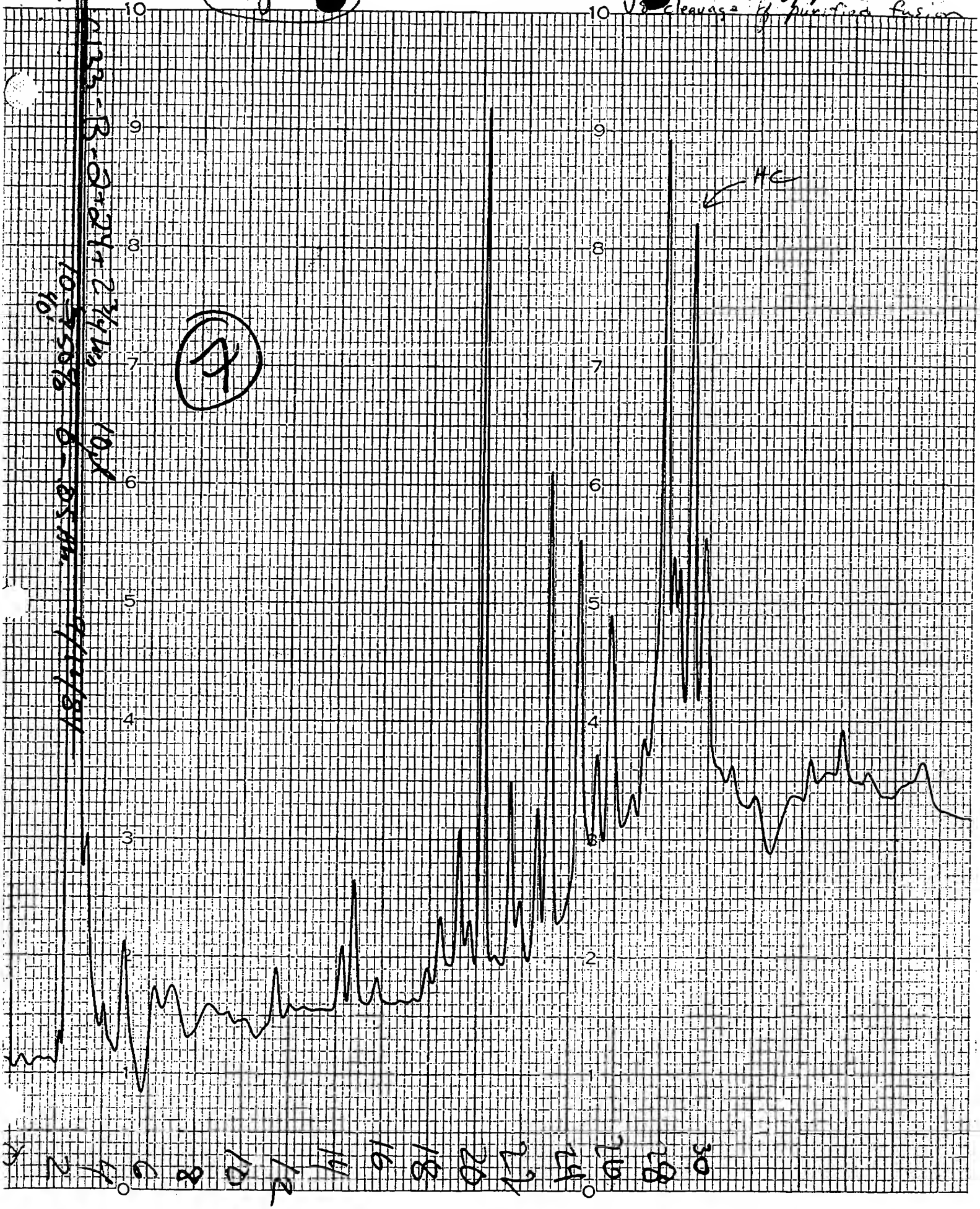
Read and Understood By

Peter A. Babiskas C.H.

EXHIBIT C

Figure

TRP-LE-calcitonin polypeptide 1
Vs cleavage of purified fusion



TRPLE- calcitonin polypeptide 2 U8 cleavage of unified fusion

Figure 2

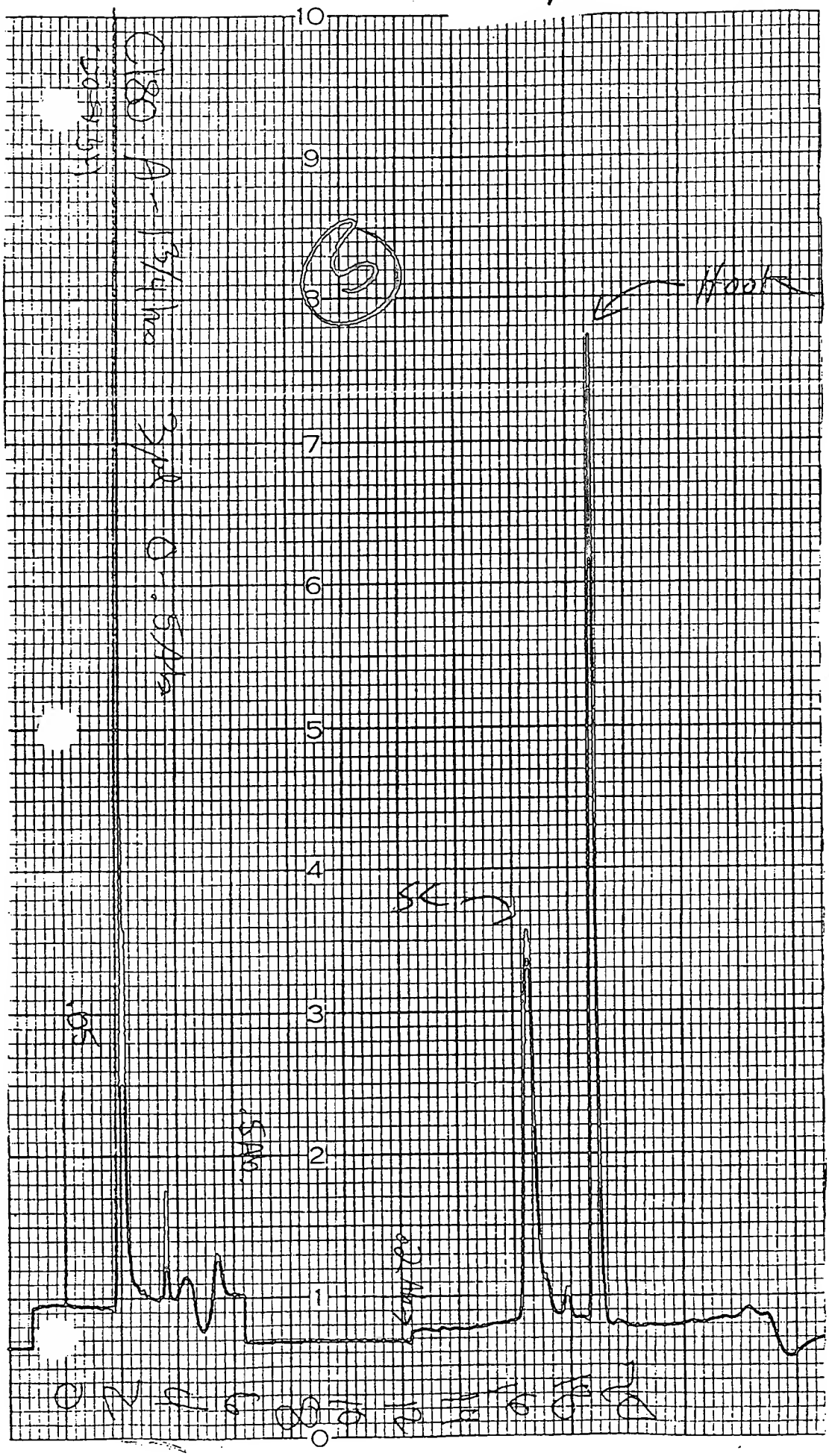


EXHIBIT E

Figure 3

polypeptide 2
V8 cleavage
purified calcitonin